NOVEL DOUBLE LOADED TRANSFEROSOMES: EVIDENCE OF SUPERIOR ANTI-INFLAMMATORY EFFICACY- A COMPARATIVE STUDY

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ABSTRACT
The main aim of this study was to enhance the transdermal penetration of a poorly water-soluble NSAID, Piroxicam, by encapsulating it into a nanocarrier namely transferosomes. In order to increase the drug encapsulation into the transferosomes, water soluble cyclodextrin complex solutions of drug were entrapped into the aqueous phase of stable unilamellar transferosomes in addition to incorporating into the liposomal bilayer by the thin film hydration method and sonication. TEM measurements suggested that, the drug cyclodextrin complexes were successfully loaded into the transferosomes without affecting their morphology. At optimal conditions, the mean particle size of double loaded transferosomes was ~158.7 nm and entrapment efficiency of ~85.7% was achieved. Addition of surfactant provides adequate fluidity to the bilayer which was further confirmed by DSC and FTIR of skin. The entrapment efficiency was increased when compared with the batches not containing the cyclodextrin complexes. The optimized formulation shows no interaction with skin components which was confirmed by skin-vesicles interaction and ex-vivo corrosion studies. Transferosomes containing cyclodextrin inclusion complexes released more drug than transferosomes encapsulating non-complexed drug, the drug release being related to entrapment efficiency. Ex-vivo lipid peroxidation studies revealed that double loaded Piroxicam transferosomes showed two times effectiveness in preventing free radical damage when compared with non-complexed drug loaded transferosomes, while it showed almost four times effectiveness against the solution of non-complexed drug in distilled water. The formulation also demonstrated superior anti-inflammatory activity in rodents compared through in-vivo carrageenan induced rat paw edema model.

INTRODUCTION
Conventional pharmaceutical dosage forms which are widely administered dermally are gels, creams and ointments. Transdermal use of carbopol gels is beneficial as they possess good rheological properties resulting in long retention time at the site of administration and high drug concentration on skin. Different approaches have been performed to enhance the transdermal passage of drugs to overcome the low skin permeability. Topical delivery of drugs formulated in lipid vesicle forms attracts considerable attention. Liposomal formulations are widely used in the pharmaceutical field as drug delivery systems due to their flexibility and clinical efficacy. Topical liposome formulations act as a solubilizing matrix for poorly water soluble drugs, as penetration enhancer and simultaneously a local depot which can be more effective and less toxic in comparison with conventional formulations. The liposomal gel formulations can perform better therapeutic effects than the conventional formulations, whereas their prolonged and controlled release property may lead to improved efficiency and better patient compliance. However, in most cases it has been shown that conventional liposomes, because of high drug deposition in the upper layers of the skin and low penetration into the deeper layers, have low efficiency as a carrier in transdermal drug delivery.

Hence a new class of liposomes also called as Transferosomes® have been developed. Transferosomes are promising nanocarriers for non invasive transdermal delivery. Better drug delivery by transferosomes, due to the driving force provided by the osmotic gradient between outer and inner layer of stratum corneum [1], thus, can pass through the intact skin spontaneously under the influence of the naturally occurring in vivo transepidermal hydration gradient. These nanocarriers possesses an edge activator (EA) such as sodium deoxycholate, span 80 or tween 80 which provides stress dependant adaptability to these carriers so that they can easily squeeze between the pores of the stratum corneum. Also, transferosomes are colloidal carriers which are easily accumulated into the leaky synovial tissue which leads to peripheral targeting. Transferosomes also act as depot resulting in controlled drug delivery system. Cyclodextrin complexation of drug increases the solubility and stability as well as sustains the drug release. Inclusion of piroxicam in cyclodextrin could possibly reduce the problem of poor aqueous solubility, low penetration; poor incorporation in vehicle associated with currently used topical formulation. Recently, encapsulation of drug in the form of cyclodextrin-drug complex in vesicular formulation has been investigated as new strategy for merging the relative advantages of the two types of carrier into a single system. In particular, the focus of investigation was to see that combination of vesicular approach with cyclodextrin complexation would help in increasing the solubility, skin permeation and deposition.

Piroxicam, a non-steroidal anti-inflammatory drug (NSAID), are used in the treatment of various acute and chronic musculoskeletal disorders like rheumatoid arthritis, osteoarthritis etc., and also as potent analgesics. However, the use of piroxicam has been associated with a number of gastrointestinal disorders [2]. Dermal delivery is an alternative route, but requires a formulation which ensures deep skin penetration. Thus the study encompasses the ability of lipid vesicles to deliver piroxicam across skin in order to evaluate its transdermal delivery potential as well as overcome its side effects.

MATERIALS AND METHODS
Drug and chemicals
Commercial grade Piroxicam (PX) was a gift sample obtained from Ranbaxy chemicals, Mumbai. Phosphatidyl choline (Phospholipon 90G (SPC)) was a gift sample from Lipoid, Germany. Beta cyclodextrin (BCD) and hydroxyl propyl beta-cyclodextrin (HP-BCD) were a kind gift from Gangwal chemicals, Mumbai, India. Sodium deoxycholate (SDC) was obtained from S.D Fine chemicals. All other chemicals were locally procured and solvents of Merck grade were used. Albino Wistar rats used in the study were purchased from Haffkine’s institute (Mumbai, India).

Preparation of Piroxicam Cyclodextrin complexes
Selection of cyclodextrin derivative
In order to find the suitable cyclodextrin that can be complexed with PX with increased solubility, phase solubility studies were carried out with two cyclodextrins i.e β-cyclodextrin and HP-BCD.

Phase Solubility studies
Phase–solubility measurements were carried out according to the method of Higuchi and Connors 1965[3]. Excess amount of PX (10
mg) was added to 10ml of deionized water containing increasing amounts of cyclodextrins (ranging from 0 to 0.020 M). The resulting mixture was equilibrated by placing the flasks on the rotary shaker at R.T for 48hrs. To minimize photochemical degradation flasks were covered with aluminium foil. Then, suspensions were filtered through 0.45 µm cellulose acetate membrane filter to remove undissolved solid. An aliquot from each vial was adequately diluted and spectrophotometrically analyzed at 358 nm. Shaking was continued until three consecutive experiments yielded similar results.

The apparent stability constant ($K_s$) of the complexes were calculated from the phase–solubility diagrams according to equation:

$$K_s = \frac{\text{Slope}}{S_0 (1 - \text{slope})}$$

Where $S_0$ is the solubility of PX at 30°C in absence of cyclodextrin and slope means the corresponding slope of the phase–solubility diagrams, ie, the slope of the drug molar concentration versus CDs molar concentration graph.

Method employed

Solid-state Piroxicam complexes with β-CD / HP-βCD in 1:1 molar ratios were prepared according to the kneading method described by Nagai et al; 1975 [4], with some modifications. Required quantity of β-CD / HP-βCD were wetted in a mortar with hydroalcoholic solution (distilled water: methanol 1:1) until a paste was obtained. The required amount of Piroxicam was then slowly added and the slurry was kneaded for about 60 min. During this process an appropriate quantity of hydroalcoholic solution was added in order to maintain a suitable consistency. The paste was dried in a vacuum oven and then sieved through 60 mesh sieve and stored away from light.

Formulation of transfersomes (PX-TRS)

Rotary evaporation-sonication method was found to give more stable transfersomal formulation. Hence this technique was chosen for formulation development and batch processing. Phospholipon 90G, PX and edge activator (surfactant) i.e SDC were dissolved in chloroform and methanol in a round bottom flask (RBF). The ratio of chloroform to methanol was 2:1 v/v. The solvent was evaporated and a thin film was formed using a rotating flash evaporator (PBU-6, Superfit, and Mumbai, India). The film was hydrated using water as hydrating medium for 1 hr at room temperature which resulted in multilamellar vesicles. These were further size reduced by ultra sound cavitation using probe sonicator (Oscar, Japan) to form small unilamellar vesicles.

Preparation of Double Loaded Transfersomes (TRSD)

Essentially, the same method of thin film hydration and sonication as stated was used to prepare transfersomes. However, while hydrating the lipid film with buffer, the PX-HP-βCD complexes were dissolved in the water and then incorporated in to the aqueous phase of the transfersomes [5]. Thus Piroxicam was “double” loaded into the lipid bilayer as well as the aqueous compartment of the transfersomes. The suspension form of the formulation was stored in air tight container at 2-8 °C for further use. For the solid state analysis, the suspension was converted into freeze dried product for further analysis.

Preparation of transfersome enriched gel

Viscosity of the transfersomal dispersion was low. Hence to achieve the desired rheological characteristics and texture for transdermal application, the optimized dispersion was converted into a gel. Various gelling agents like Carbopol Ultrez 10 and Carbopol 940 were evaluated for their gelling ability. Based on the compatibility with nanoparticulate dispersions, feel, aesthetic appeal and ease of spreadability Carbopol Ultrez 10 was selected as the gelling agent.

Gel dosage forms of PX were prepared using a serial mixture of deionized water and glycerin in the ratio of 4.5:0.5 w/w as the vehicle and Carbopol Ultrez 10. Different concentrations of Ultrez 10 ranging from 0.5-1% w/w were used for gelling and the concentration giving the optimum viscosity was chosen for further studies. For the final formulation 0.75% w/w Ultrez 10 was selected and dispersed into the vehicle to give the total drug concentration of 0.25% w/w.

Triethanolamine was added to adjust the pH to 7, and then remaining vehicle was added to give a total weight of 20 g. Gel was dispersed thoroughly using an overhead stirrer at the speed of 800 rpm (Remi, Mumbai, India) for 3h.

Physico-chemical characterization

The vesicle size and distribution were determined by dynamic light scattering method Malvern zetasizer (Malvern Instruments Ltd.). Measurements were carried out at an angle of 90° at 25°C.

Dispersions were diluted with double distilled water to ensure that the light scattering intensity was within the instrument’s sensitivity range. For the imagine analysis by TEM, copper grids having a thin layer of carbon was loaded with drug loaded transfersomal dispersion and allowed to dry at room temperature. After the sample was dried thoroughly, the images were captured on a Philips Tecnal-20 Transmission Electron Microscope (Philips, Holland) with 0.27nm point resolution and accelerating voltage of 200 Kv. For zeta potential analysis, a proper amount of PX transfersomes were diluted appropriately and determined by Malvern particle size analyzer. For determination of entrapment efficiency the unentrapped drug was separated by centrifugation method. The amount of drug entrapped (Total amt. of drug- unentrapped drug) in the vesicles was then determined by disrupting the vesicles using methanol followed by filtration and amount of PX was quantified spectrophotometrically at 358 nm.

Percentage entrapment = \frac{\text{Amount of Entrapped PX} \times 100}{\text{Total PX added}}

In vitro drug release from transfersomes

In vitro drug release was evaluated using Keshary chein diffusion cells. A cellophane dialysis membrane with molecular weight cut-off of 8000 Da (Hi-media) was hydrated with the receptor medium with phosphate buffer pH 7.4(PBS 7.4) overnight before being fastened between the donor and receptor compartments. The donor medium consisted of 2 ml of the transfersomal dispersions. The same was repeated with dispersion loaded gels. The receptor compartment was filled with 13 ml of PBS 7.4 and stirred with a magnetic bar. The available diffusion area was 2.61 cm². The temperature of the assay was controlled at 32 °C to mimic human skin. 2ml aliquots were withdrawn at fixed time intervals and immediately replaced with an equal volume of fresh buffer. All samples were analyzed for PX content by spectrophotometry at 358 nm. The experiment was done in triplicate.

In vitro skin permeation study

Porcine ear skin from local slaughter house was used as a model membrane for the skin permeation study because of its similarity to human skin in lipid content and permeability. The skin samples were mounted on the keshary chein diffusion cell and the temperature was maintained at 37°C. The dorsal surface of the skin was placed in contact with the donor chamber, which was filled with the transfersome formulation. The receptor chamber was filled with PBS 7.4 and stirred with a star-head Teflon magnetic bar driven by a synchronous motor. At time intervals of 0.5, 1, 2, 4, 8 and, 24 h, 1ml aliquot of receptor was withdrawn, and replaced by fresh medium. The concentration of drug in the samples was analyzed spectrophotometrically at 358 nm, and the cumulative amount was plotted against time. The steady state flux was determined as the slope of linear portion of the plot.

Ex-vivo lipid peroxidation studies

Preparation of Liver Homogenate

- The albino wistar rats (weighing 100-150gm, overnight fasted before experiment) were sacrificed
- The liver was quickly removed and chilled in ice cold saline.

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• After washing with ice cold saline, the liver was homogenized in 0.15 M KCl to get 10% liver homogenate.

Methodology:
The method proposed by Ohkawa et al, 1979 [6] was used for estimation of inhibition of lipid peroxidation.

• Fresh 0.2 ml of liver homogenate was mixed with 150 mM of 0.1 ml KCl and 0.4 ml of TRIS buffer. The test samples of 0.1 ml of plain PX, TRS and PX-TRSDS gel solution were then added in various concentrations.

• In vitro lipid peroxidation was initiated by addition of 0.1 ml each of FeSO₄(10μM) and 100 μl of test samples.

• After incubation for 1hr at 37°C, reaction was terminated by addition of Thiobarbituric acid (TBA) 2 ml of reagent and boiled at 95°C for 15 mins for development of colored complex.

• After cooling, the tubes were centrifuged at 4000 rpm for 10 mins. The absorbance of supernatant was determined colorimetrically at λmax of 532 nm.

• Percentage inhibition of TBA reacting substances (TBARS) formation was calculated with respect to control in which no test sample was added. The inhibition of lipid peroxidation was determined by calculating the % decrease in the formation of TBARS and IC₅₀ was calculated.

Calculation:
The percent inhibition of lipid peroxidation of test/standard PX was calculated by following equation:

\[
\text{% Inhibition} = \left( \frac{A_0 - A_t}{A_0} \right) \times 100
\]

Where, \( A_0 \) is the absorbance of the control (blank) and \( A_t \) is the absorbance of the test samples.

In vivo studies (pharmacodynamics) by carrageenan paw edema test
All studies were carried out in accordance with the principles of Laboratory Animal Care and the experimental protocols were reviewed and approved by the Institutional Animal Ethics Committee (Animal House Registration No. 25/1999/CPCSEA). The anti-inflammatory activity of the gel formulations was studied by carrageenan induced rat paw edema volume model. The rats weighing 150–180 g were randomly divided into 4 groups of six rats each. An identification mark was made at the ankle joint of each animal. PX-TR5, PX-TRSDS gel and standard gel were applied on the subplantar region of the left hind paw of first, second, and third groups respectively. Fourth group was untreated and served as control 1 h post transdermal application, paw edema was induced by subplantar injection of 0.1 ml of a 1% w/v freshly prepared carrageenan in normal saline into the left hind paw of each rat. The paw volume up to the ankle joint was measured before and at different time intervals after the carrageenan injection using graduated plethysmograph (INCO, India). All the test groups were compared with control group. Percentage reduction in edema was calculated using the following formula [7].

\[
\text{% Inhibition of paw edema} = \left( \frac{V_0 - V_t}{V_0} \right) \times 100
\]

Where,

\( V_0 \) is the paw volume at time‘

\( V_t \) is the initial paw volume (before carrageenan treatment)

\( V_0 \) control is edema produced in control group

\( V_t \) treated is edema produced in treated group

Ex vivo skin corrosion studies
The human skin was exposed to a great amount of different chemicals that need to be classified according to their capacity to harm the skin. The product is defined corrosive if the skin is reversibly damaged, whereas the product is defined as irritative, if the skin is reversely altered. Assays were developed based on tissue-engineered living skin substitutes. In order to determine the corrosive potential of transferosomal dispersions, an ex vivo method reported by Padois et al; 2009 [8] was adopted. The method has been developed from the Corrositex® test. Corrosive substances are able to destroy the epidermis proteins and lead to a color shift in an underlying chemical detection liquid. Corrosive potential of formulations was determined on pig ear skin. 37% nitric acid and 0.9%/w NaCl solutions were used as positive and negative control, respectively. Skin punches were prepared and clamped on Franz-type glass diffusion cells. 200μl of 37% nitric acid solution or studied sample was deposited onto the epidermis. After 15 min, 200μl was removed. Epidermis was washed with 2 x 1 ml of distilled water to remove the residual sample. 1ml of Sulforhodamine B (skin proteins labeling dye) was deposited onto the epidermis. After 15 min, the 1ml of Sulforhodamine B was removed. Then epidermis was washed with 1 ml of distilled water. The absorbance (Abs) of the washing water was measured with a spectrophotometer (Jasco, Japan) at 313 nm. The corrosive factor was calculated with the Eq.

\[
F = \frac{\text{Sample Abs}-0.9\% \text{ NaCl solution Abs}}{0.9\% \text{ NaCl solution Abs}}
\]

If \( F > 0 \), then the sample is non-corrosive. If \( F < 0 \), then the sample is corrosive.

The experiment was conducted in triplicate.

Drug deposition studies on skin
Skin deposition study was carried out using same protocol as discussed above for skin permeation study. At the end of the permeation experiments (24 h), the surface of the skin was washed five times with 50% ethanol to remove excess drug from the surface. The washing protocol was verified and found to remove >95% of the applied dose at zero time. The skin was then cut into small pieces. The tissue was further homogenized with 50% ethanol (10 ml) and left for 24 h at room temperature. After shaking for 5 min and centrifugation for 5 min at 3,000 rpm, the PX content in the upper phase was determined by UV [9].

Evaluation of bilayer fluidity of stratum corneum (SC) using FTIR spectroscopy
Stratum corneum was cut into small circular discs. 0.9% w/v solution of sodium chloride was prepared and 0.01% w/v sodium azide was added as antibacterial and antifungal agent. 30 ml of 0.9% w/v of sodium chloride solution was placed in different conical flasks and SC of approximate 1.5 cm diameter was floated over it for 3 days. After 3 days of hydration, these discs were thoroughly blotted over filter paper and FTIR spectra of SC discs was recorded before transferosomal gel treatment (control) in frequency range of 400 to 4000 cm⁻¹ (Shimadzu, Japan) using KBr as the substrate. After taking FTIR spectra, the same discs were dipped into transferosomal gel formulation. This was kept for a period of 48 h (equivalent to the permeation studies) at 37 ± 2°C. Each SC disc after treatment was washed, blotted dry, and then vacuum dried for 3h. FTIR spectra of treated SC discs were recorded again.

Evaluation of bilayer fluidity of stratum corneum (SC) using differential scanning calorimetry (DSC)
Thermal analysis of the skin was done using the same skin sample which was used for permeation analysis. The skin sample (2 mg) was weighed into an aluminum crimp pan. The samples were heated from –30 to 320°C at a heating rate of 10°C/min. All DSC measurements were collected under a nitrogen atmosphere with a flow rate of 100 ml/min.

Vesicle skin interaction studies by light microscopy
Skin irritancy and toxicity potential of the formulation upon application of the TDDS containing gel formulation, with and without drug was carried out by preliminary histopathological evaluation. After 48 h of application, skin was removed from the
diffusion cell and stored in 10% formalin solution in phosphate buffer saline (pH 7.4) followed by dehydration with alcohol. It was then treated with antimedia and embedded in paraffin for fixing. Sections of ~5 μm thickness were cut from each skin piece and stained with hematoxylin and eosin. These samples were then observed under light microscope (Leica, Germany) and compared with control sample for structural changes of epidermis [10].

RESULTS

Selection of cyclodextrin derivative by Phase solubility Studies:
The complexation of PX with β-CD and HP-BCD was investigated by phase solubility studies. The aequous solubility of PX was found to increase linearly as a function of the concentration of CD. The phase solubility diagrams of PX-CD complexes can be classified as type AL according to Higuchi and Connors. Because the straight line had a slope<1 in each case, the increase in solubility was due to the formation of 1:1 M complex in solution with both β-CD and HP-BCD .It was reported that HP-BCD gave higher enhancement in the dissolution rate and efficiency of PX when compared to BCD [11]. It was observed that drug permeated faster from transferosomes containing Drug-HP-BCD than from Drug-βCD complex. Hence, HP-BCD was selected for further studies.

Table 1: It shows stability constant [Kc] of Cyclodextrins with Piroxicam:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Kc</th>
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<tbody>
<tr>
<td>Pxn-β-CD</td>
<td>304.13</td>
</tr>
<tr>
<td>Pxn-HP-β-CD</td>
<td>683.5</td>
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</table>

Formulation of Double Loaded Transferosomes

The optimised batch TRS5 was chosen for loading of the cyclodextrin-drug complex into the transferosomes. The double loaded batch was subsequently subjected to further characterization and transformed into gel form. Influence of CDs on the bilayer stability is of major concern. Bouldemarat et al; 2004 [12] have reported that the CDs, especially the methylated forms, are known to destabilize the lipid membrane of the transferosomes. However, for CDs to effectively function as a destabilizing agent, it should be present in the free form. In our system, it is present as a 1:1 stable complex with Piroxicam, and this complex has an appreciable stability constant of 683.5/M with HP-BCD. Results demonstrate that liposome integrity in cyclodextrins is affected by lipid composition and type. It was observed by Hatzi et al; 2007 [13] that for the same lipid composition calcitoxin release from vesicles is faster in the order: MLV>DVR> SUV. A plausible explanation for the high stability demonstrated by SUV transferosomes, which were only affected by Me-β-CD [the most lipophilic of the CD molecules used], maybe that curvature of the vesicle surface does not permit initial interaction between membrane components and CD molecules, for the CD molecules that interact with the transferosomes only by surface contact [13]. Hence, the possibility of instability occurring in the system during storage is ruled out, if the system is adequately freeze-dried and stored appropriately under low temperatures. Hence freeze drying was carried out using trehalose as cryo-protectant to ensure stability.

Particle size analysis

The small droplet size of transferosomes provides a very large surface area for drug transfer into the skin [14]. In addition particle size of the double loaded transferosomes showed that no significant difference as compared to the single loaded ones which was confirmed by TDM as depicted in fig 2. Polydispersity index were in the range of 0.224-0.516 ensuring good stability of the formulation.

Zeta potential analysis

The zeta potential of optimized batch was found to be -22.7mV which is in agreement with literature due to the net charge of the lipid composition in the formulations. PC is a zwitterionic compound with an isoelectric point [pI] between 6 and 7. Under experimental conditions (pH 7.4), where the pH was higher than its pI, PC carried a net negative charge. The edge activators used were anionic edge activators, and the anion form of PX was also the predominant form at pH 7.4. Therefore, a negative charge in all formulations was observed. Also the negatively charged liposome formulations strongly improved skin permeation of drugs in transdermal delivery [15]. The skin has also slight negative charge. Therefore, the negative zeta potential of the optimized transferosomal gel containing drug might cause little influence in improved drug permeation through porcine skin due to electrostatic repulsion between the same charge of the skin surface and the optimized gel. [16]

Drug entrapment efficiency

Entrapment Efficiency of double loaded transferosomes PX-TRS5 showed highest entrapment efficiency of 85.7%. Here, the amount of surfactant was 2% w/w. This increase in encapsulation efficiency could be explained by the presence of sodium deoxycholate in the bilayer, which could be supposed to “solubilise” and “hold” the free PX [PX-HPBCD complex is in equilibrium with free PX and cyclodextrin] in the lipid bilayer and therefore enhance the encapsulation efficiency for the transferosomes, which correlates by the work done by Chen et al; 2009 [17] with fenofibrate.

TEM

TEM images showed that the transferosomes are spherical in shape, but slightly differ in size. The size of the double loaded transferosome is about 157.8 nm, as shown in Fig. 2.

The inner phase constitutes the reservoir [aqueous core] of the system which contains the embedded drug-cyclodextrin complex. TEM images reveals formation of unilamellar vesicles with the bilayer membrane intact. This shows that the inclusion of cyclodextrin-drug complex within the liposome did not affect the bilayer and lamellar integrity.
Evaluation of bilayer fluidity of stratum corneum [SC] using FTIR spectroscopy and DSC

FTIR spectroscopy is a non-invasive technique for characterization of SC at a molecular level. FTIR spectrum of untreated SC [control] showed various peaks due to molecular vibration of proteins and lipids present in the SC. The absorption bands in the wave number of 3000 to 2700 cm\(^{-1}\) were seen in untreated SC. These absorption bonds were due to the C-H stretching of the alkyl groups present in both proteins and lipids. The bands at 2978.39 cm\(^{-1}\) and 2880 cm\(^{-1}\) were due to the asymmetric \(-\mathrm{CH}_2\) and symmetric \(-\mathrm{CH}_2\) vibrations of long chain hydrocarbons of lipids respectively. The bands at 2949.16 and 2870.08 cm\(^{-1}\) were due to the asymmetric and symmetric \(\mathrm{CH}_3\) vibrations respectively. These narrow bands were attributed to the long alkyl chains of fatty acids, ceramides and cholesterol which are the major components of the SC lipids. The two strong bands [1649.14 cm\(^{-1}\) and 1544.98 cm\(^{-1}\)] were due to the amide I [C=O] and amide II [C-N] stretching vibrations of SC proteins. The amide I band consisting of components bands, represented various secondary structure of keratin. The skin band for \(\mathrm{CH}_2\) scissoring mode was also present in the wave number region of 1470-1450 cm\(^{-1}\). The band for C=O stretching from fatty acids were present at 1786.08 cm\(^{-1}\).[14]

Of particular interest for studying the interaction of the formulations with SC lipids are \(\mathrm{CH}_2\) stretching bands. Analyses of these bands provide information about conformational order [trans-gauche isomerization] of the lipid alkyl chains [18]. After the treatment of dermatomed porcine skin with transferosomal formulations, symmetric and asymmetric methylene stretching bands were shifted to significantly lower values compared with the control samples, indicating a lipid order–disorder transition from the gel to the liquid–crystalline state. This transition also involves an increase in the fluidity of the SC lipids that could increase the skin penetration. [19]

The region corresponding to \(\mathrm{CH}_2\) scissoring vibration provides information about the lateral packing of lipid alkyl chains in the SC. In all skin samples at both temperatures [37 °C and 45 °C], \(\mathrm{CH}_2\) scissoring band displayed a doublet, characteristic of the orthorhombic [OR] lattice, which is very important for the barrier function of the lipids. This doublet is caused by short-range interaction between the \(\mathrm{CH}_2\) in the lipid tails [20]. The split of the band is indicative of the degree of inter-chain interaction and the size of the domain with -OR organization. The treatment with transferosomal formulations showed slight increase in width of the doublet at both temperatures. In conclusion, the FTIR data suggest that the SC barrier function is partially overcome by transferosomal application. [21].

Fig. 2: It shows TEM image of Double loaded Piroxicam Transferosomes Batch TRSD [Bar=100nm]

Fig. 3: FTIR spectra of [A] Untreated Porcine Skin [B] Double loaded transferosomal gel [TRSD5 gel] treated skin
DSC

Porcine stratum corneum samples were also investigated by DSC techniques. The results obtained were remarkably similar to thermal profiles of human samples. This may be due to the similar lipid composition. In these experiments it was proposed that the transitions occurring near 60°C and 75°C, in control hydrated samples, were due to intercellular lipid, protein intercellular lipid and keratin respectively [22]. The DSC profiles are compared in Fig 4. Treatment with PX-TRSD5 resulted in a pronounced shift to lower temperature. As can be seen, the maximal thermal transition temperature is shifted from about 60°C to 54°C after treatment with PX-TRSD5. This indicates an interaction with the skin lipids. As seen with other penetration enhancers, like monounsaturated acids, the findings also suggest that incorporation transferosomes into stratum corneum results in a decreased lipid order. This is in agreement with the work carried out by Duangjit et al; 2011 [23].

![Fig 4: DSC thermogram of [A] Untreated Porcine Skin [B] Double loaded transferosomal gel [TRSD5 gel] treated skin](image)

**Ex-vivo corrosive studies**

The corrosive factor was calculated with the Eq.

\[ F = \frac{[\text{sample Abs} - 0.9\% \text{NaCl solution Abs}]}{[0.9\% \text{NaCl solution Abs}]} \]

If \( F > 0 \), then the sample is non-corrosive. If \( F < 0 \), then the sample is corrosive. The experiment was conducted in triplicate. The results are shown in Table 2.

**Table 2: It shows corrosive factor of formulations**

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Corrosive Factor</th>
</tr>
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<tbody>
<tr>
<td>37% Nitric acid solution [positive control]</td>
<td>-1.334 ± 0.003</td>
</tr>
<tr>
<td>PX-TRS</td>
<td>1.299 ± 0.002</td>
</tr>
<tr>
<td>PX-TRSD</td>
<td>1.330 ± 0.002</td>
</tr>
</tbody>
</table>

As proved from the result, the both TRS dispersions were found to be non-corrosive as compared to positive control.

**In vitro diffusion studies**

The diffusion studies were carried out in non-occlusive conditions to allow the driving force provided by the osmotic gradient. As expected, encapsulation of Piroxicam into transferosomes led to controlled release rate due to the well known reservoir effect of transferosomes, with the release profile being identical in all cases. The results indicate that, transferosomes containing Piroxicam-Beta cyclodextrin complex release more drug than do transferosomes encapsulating non-complexed drug, drug release being related to entrapment efficiency and drug loading.

![Fig 5: In-vitro release of Piroxicam from [a]plain solution [b] std. marketed gel [c]plain drug loaded transferosomes [d] double loaded transferosomes [e] gel formulation](image)
It may be possible that during complex entrapment in transfersomes, phospholipid enters the hydrophobic cavity of the empty cyclodextrin molecules present in the transfersomes’ aqueous phase, or perhaps replaces the drug when the cavity contains drug molecules.

Perhaps, Piroxicam may be released as both free forms as well as in the complexed form from the aqueous compartment. This release would then be attributed to the dissociation of Piroxicam from the complex followed by its partitioning into the lipid bilayers at rates dependent upon its partition coefficient. [24].

It was observed that the sustained effect in the order of double-loaded transfersomes > non-complexed drug-loaded transfersomes > standard marketed gel > plain drug. While the lower solubility of the plain drug can account for its lower cumulative release, the release pattern of both the plain drug-loaded transfersomes and the CD-loaded transfersomes are almost similar, suggesting that double loading does not interfere with the release pattern of Piroxicam. The improved release of the double-loaded liposome in lieu of the plain drug-loaded liposome can then be conveniently attributed to the higher drug: lipid ratio in the former [25].

The release profile of PX-TRSD enriched gel indicated slow release as compared to PX-TRSD dispersion. This can be explained by the fact that drug diffusion from the transfersomal carrier followed by diffusion from the gel matrix resulted in sustained release effects.

**Ex vivo skin permeation analysis and drug deposition studies**

The *ex vivo* permeation studies provided valuable information about the product behaviour in vivo since they indicate the amount of drug available for absorption. The action of transfersomes as penetration enhancer may predominantly be on the intercellular lipid of stratum corneum, raising the fluidity and weakness of stratum corneum. Ultradeformable character of transfersomes supports their passage through very fine pores in the skin under suitable osmotic gradient. Phospholipids have high affinity for biological membranes. Mixing of the phospholipid of the carrier system with the skin lipid of the intercellular layers may also contribute to the permeability of the skin to lipid vesicles. The presence of unsaturated fatty acid in SPC may be responsible for enhanced permeation. The packing nature of unsaturated fatty acids changed the fluidity of stratum corneum lipid structure and facilitated the permeation of bioactive [26]. Topically applied lipid vesicles affect characteristics and integrity of the skin permeability barrier. In addition, they may extract the lipid from the skin or disrupt the order within and between the corneocyte upon binding to the keratin filament. Elastic vesicle can be used to transfer bioactive rapidly into the deeper layer of the stratum corneum, after which the bioactive can permeate into the viable epidermis. This is well supported by the study carried out by Gupta et al; 2005 [27].

![Fig. 6: Ex vivo skin permeation analysis of Piroxicam from (A) Std. Marketed Gel (B) Plain (Non-Complexed) Transfersomal Gel (C) Double Loaded Transfersomal Gel](image)

The accumulated permeation amount in 24h was up to 64% for double loaded transfersomal gel which was obviously higher than that of plain drug and conventional gel. It can be corroborated that the presence of sodium deoxycholate contributed to deformation infiltration of transfersomes. Therefore, the transfersomes had a good ability of transdermal permeation. Transdermal fluxes through the skin from the formulations were calculated by plotting the cumulative amount of drug permeating the skin against time and determining the slope of the linear portion of the curve by linear regression analysis. The permeability coefficient \([K_p]\) was obtained by dividing the flux \([J]\) by the initial drug concentration \([C_0]\) in the donor phase:

\[
K_p = \frac{J}{C_0}
\]

**Table 3: It shows permeability coefficient and flux for the formulations assayed**

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Permeability coefficient ([\times 10^{-2}]) ([\text{cm}^2 \text{h}^{-1}])</th>
<th>Flux ([\mu g \text{h}^{-1} \text{cm}^{-2}])</th>
</tr>
</thead>
<tbody>
<tr>
<td>Std marketed gel</td>
<td>0.1486</td>
<td>7.43</td>
</tr>
<tr>
<td>Double loaded transfersomes</td>
<td>15.68</td>
<td>23.53</td>
</tr>
</tbody>
</table>

The activity of penetration enhancers may be expressed in terms of an enhancement ratio \([\text{ER}]\):

\[
\text{ER} = \frac{\text{Drug permeability coefficient [Treated]}}{\text{Drug permeability coefficient [Control]}}
\]

The enhancement ratio of the double loaded transfersomes was found to be 10 times more as compared to that of a conventional gel. As compared to non-complexed drug loaded transfersomes, ER was found to be approximately 5 times more.
In case of drug deposition studies, TRSD5 gel formulations showed desirable rheological properties, which will ensure its sufficient long contact time with the skin thus having better skin deposition but due to intact network of the carbopol, the release of the transferosomal vesicle is sustained as compared to TRS5 dispersion [28]. Therefore, despite the fact that transferosome dispersions deliver higher Piroxicam amounts into the skin, this study showed that it is also possible to deliver a sufficiently high drug amount into the skin in a sustained and controlled manner by using a transferosomal gel as compared to simple hydro-gel and aqueous drug solution.

**Fig. 7:** It shows comparative study of Piroxicam skin deposition [%] of various formulations.

**Fig. 8:** It shows lipid peroxidation inhibition % of PX TRS5, TRSD5, Data expressed as Mean± SEM

**Ex vivo lipid peroxidation studies**

Increased level of ROS has been implicated in the pathogenesis of rheumatoid arthritis. Excessive ROS production disturbs redox status, damages macromolecules, including DNA and can modulate expression of a variety of immune and inflammatory molecules leading to inflammatory processes, exacerbating inflammation and affecting tissue damage [29]. Several chemokines are capable of triggering free radical generation at the site of inflammation by the activated neutrophils in the inflammatory disease. These observations added another link to the relationship between ROS and chemotactic chemokines production in the autoimmune diseases. Chemokines and oxidative stress in rheumatoid disease and hypothesized play an important role in recruiting and activating leukocytes and in enhancing oxidative stress in these diseases. Controlling the ROS in RA patients can be effective in preventing the cartilage destruction. Inhibition of oxygen radical overproduction in RA by antioxidants can be considered as a useful supporting treatment regime for free radical pathologies. Selective COX-2 inhibitors are most promising therapeutic approach for RA.

Overproduction of free radicals causes a chain reaction of peroxidation on the cell membrane lipid that could lead to cell and tissue death. The inhibitory effect on ferric ion-vitamin C system-induced lipid peroxidation in rat liver homogenate was used to assess the anti-lipid peroxidation activity of the different drug systems. Piroxicam loaded transferosomes elicited concentration dependent inhibition of FeSO4 induced lipid peroxidation in rat liver homogenate. IC50 value is indicative of the concentration of the drug that is required to elicit 50% of the proposed activity. The IC50 value of batch TRS5 was found to be 45.04 ± 18 μg/ml and that of TRS5 95.34 ± 12 μg/ml whereas plain drug showed an IC50 value of 140.4 ± 33 μg/ml only as shown in Fig. 9. Thus, TRS5 showed better inhibition of lipid peroxidation. In other words, almost double the quantity of TRS5 is required to elicit the same response as that produced by TRSD5. As compared to drug in distilled water TRSD5 showed four times increase in activity. There is a significant difference between the tested groups and the control as determined by one way ANOVA with p < 0.05.

**Pharmacodynamics study**

Carrageenan induced edema as shown in Fig. 10 in paw being attributed to two phases, the initial phase is the release of histamine and serotonin, and the second phase is the release of prostaglandins and lysosomal bodies, which are sensitive to most clinically effective anti-inflammatory drugs.

The fast inhibitory action of a gel formulation at the receptor sites may be a desirable feature for anti-inflammatory drugs to induce maximal pharmacological action without being prematurely wiped off from the applied site. Previously, Mei et al. 2005 [30] have reported that the formulations that had a small particle size showed the strongest acute anti-inflammatory activity.

**Table 4:** It shows Percent (%) reduction in edema produced by PX containing transferosomal gel (TRS and TRSD5 gel), and std marketed (std gel) in carrageenan induced rat paw.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Treatment [n = 6]</th>
<th>0 hr</th>
<th>1 hr</th>
<th>2 hr</th>
<th>3 hr</th>
<th>4 hr</th>
<th>6 hr</th>
<th>24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>0.88±</td>
<td>1.27±</td>
<td>1.36±</td>
<td>1.62±</td>
<td>1.59±</td>
<td>1.52±</td>
<td>1.19±</td>
</tr>
<tr>
<td>2</td>
<td>Standard [Marketed formulation]</td>
<td>0.0060</td>
<td>0.0050</td>
<td>0.0069</td>
<td>0.0045</td>
<td>0.0050</td>
<td>0.0065</td>
<td>0.0057</td>
</tr>
<tr>
<td>3</td>
<td>TRSD-enriched gel</td>
<td>0.36±</td>
<td>1.06±</td>
<td>1.01±</td>
<td>1.03±</td>
<td>1.03±</td>
<td>1.07±</td>
<td>0.901±</td>
</tr>
</tbody>
</table>

**Fig. 9:** It shows Ex Vivo lipid peroxidation of Piroxicam Transferosomes
However, application of formulation TRS5, TRSD5 and the marketed gel produced, mean percentage inhibition of 86.39% and 60.46%, respectively, after 24 h after the carrageenan insult. This may be due to the reduced percentage of drug release in marketed piroxicam gel, which was not enough to control edema effectively for multiple hours. There is a significant difference between the tested groups and the control as determined by a one-way ANOVA with p < 0.05.

Vesicle skin interaction studies by light microscopy

In control the uppermost layer i.e. epidermis, dermis and subcutaneous tissues were clearly seen, whereas in the treated skin with PX-TRSDS after 24 h, widened and loose stratum corneum [5–6 distinct layers] with presence of empty spaces in the dermis were observed. It is also apparent that phospholipid can occlude the skin surface and thus can increase tissue hydration, thereby can increase drug permeation, justifying carrier’s proposed mechanism of penetration into the skin and further fusing with the skin lipids in the epidermis and dermis. Therefore, it was concluded that the transfersomes [PX-TRSDS] is an enhanced carrier for the transdermal drug delivery of Piroxicam.

CONCLUSION

In the present study, transfersomes loaded with non-complexed piroxicam as well HP-BCD piroxicam complexes were developed and characterized. In conclusion, our findings emphasize the superior anti-inflammatory efficacy of the prepared double loaded transfersomes. In vivo and ex vivo results indicate that vesicles, and especially double loaded transfersomes, are capable of really localizing the drug at the site of inflammation as compared to the conventional dosage forms. Thus, the developed transfersomal formulation may prove to be a promising carrier for piroxicam and other similar drugs, especially due to their simple production and ease of scale-up.

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REFERENCES


